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Eumelanin-Related Indolic Compounds in the Urine of Treated Melanoma Patients[1])

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Summary: A method was developed for the quantitation of six eumelanin-related indolic compounds in urine. The procedure employs methane ionization of the hexafluoroisopropyl and/or pentafluoropropionyl derivatives of 5,6-dihydroxyindole, 5-hydroxy-6-methoxyindole, 6-hydroxy-5-methoxyindole, 5-hydroxy-6-methoxyindolyl-2-carboxylic acid, 6-hydroxy-5-methoxyindolyl-2-carboxylic acid and 5,6-dimethoxyindolyl-2-carboxylic acid together with their deuterated analogues, and selected ion-monitoring. Deuterated analogues are added as internal standards to enzymatically hydrolysed urine before diethyl ether extraction.

The method was used for monitoring urinary excretion in patients with malignant melanoma treated with cytostatics. In several cases, a decrease of the excretion of the eumelanin-related substances was recorded after the therapy. The usefulness of the individual indolic compounds for such follow-up studies is discussed.

Eumelanin-verwandte Indolverbindungen im Harn behandelter Melanompatienten

Zusammenfassung: Eine Methode zur quantitativen Bestimmung von sechs Eumelanin-verwandten Indolverbindungen im Harn wurde erstellt. Das Verfahren verwendet die Methan-Ionisierung der Hexafluorisopropyl- und/oder Pentafluorpropionyl-Derivate von 5,6-Dihydroxyindol, 5-Hydroxy-6-methoxyindol, 6-Hydroxy-5-methoxyindol, 5-Hydroxy-6-methoxyindolyl-2-carbonsäure, 6-Hydroxy-5-methoxyindolyl-2-carbonsäure und 5,6-Dimethoxyindolyl-2-carbonsäure gemeinsam mit ihren deuterierten Analogen und Selected Ion Monitoring. Deuterierte Analoge wurden als interne Standards vor der Extraktion mit Diethylether nach enzymatischer Hydrolyse der Konjugate zum Harn zugefügt.

Die Methode wurde zur Überwachung der Ausscheidung der Verbindungen im Harn von cytostatisch behandelten Patienten mit malignem Melanom eingesetzt. In mehreren Fällen wurde nach der Therapie ein Abfall der Ausscheidung Eumelanin-verwandter Substanzen festgestellt. Die Brauchbarkeit der Bestimmung der einzelnen Indolverbindungen für solche Überwachungsstudien wird diskutiert.

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Introduction

A well known problem in the management of cancer patients is the detection of the clinically occult changes of the disease. Different chemical approaches to these problems have been attempted, particularly in those tumours that exhibit characteristic biochemical features. Since malignant melanocytes usually synthesize melanin, it is not surprising that attention has been paid to the intermediates of the melanin synthesis and their determination in urine and serum of melanoma patients.

The attempts to use the pheomelanin precursor 5-S-cysteinyl-DOPA as a tumour marker for malignant melanoma are well known (1–3). Much less is known, however, about the possibility of employing eumelanin precursors and their metabolites (eumelanin-related compounds). This group consists of seven indolic substances:

5,6-dihydroxyindole,
5-hydroxy-6-methoxyindole,
6-hydroxy-5-methoxyindole,
5,6-dihydroxyindolyl-2-carboxylic acid,
5-hydroxy-6-methoxyindolyl-2-carboxylic acid,
6-hydroxy-5-methoxyindolyl-2-carboxylic acid and
5,6-dimethoxyindolyl-2-carboxylic acid.

Two of them, namely 5-hydroxy-6-methoxyindolyl-2-carboxylic acid and 6-hydroxy-5-methoxyindolyl-2-carboxylic acid, were identified by *Duchon & Matous* in 1967 (4), the others were described during the last three years (5–7).

Gas chromatography was shown to be a useful technique, providing good separation of the eumelanin-related indolic compounds from urine of patients with malignant melanoma (5, 8). It failed, however, to detect the substances in urine samples of healthy persons, owing to their low concentrations and peak interferences with other compounds. Therefore a more selective technique of detection has been chosen.

In this paper we report our first experience with a gas chromatographic mass spectrometric method in chemical ionization mode, which has been used for follow-up of the urinary excretion of eumelanin-related indolic compounds in patients before, during and after cytostatic treatment.

Materials and Methods

Chemicals

Pentafluoropropionic anhydride and 1,1,1,3,3,3-hexafluoroisopropanol were purchased from Pierce Chemical Co., Rockford, IL, U.S.A., *Helix pomatia* juice was obtained from l'Industrie Biologique Française, Gennevilliers, France. 5,6-Dihydroxyindole, 5-hydroxy-6-methoxyindole, 6-hydroxy-5-methoxyindole, 5-hydroxy-6-methoxyindolyl-2-carboxylic acid, 6-hydroxy-5-methoxyindolyl-2-carboxylic acid, 5,6-dimethoxyindolyl-2-carboxylic acid as well as their deuterium labelled analogues were prepared as described earlier (9). The compounds were stored as diluted ethyl acetate solutions at −40 °C under nitrogen, and their actual concentrations were measured shortly before their use by gas chromatography with a flame-ionization detector.

All other chemicals were purchased from Merck, Darmstadt, West Germany.

Patients and treatment

Brief descriptions of the individual cases of the melanoma patients are given in the legends to the figures.

All the patients were treated according to the following five day chemotherapeutic scheme:

days 1–4: (a) bleomycin 30 mg/day in a continuous intravenous infusion
(b) dacarbazine 300 mg/m² & day intravenously
day 5 (a) vindesine 3 mg/m² in an eight-hour infusion
(b) actinomycin D 2 mg/m² in a half-hour infusion

To improve the alimentary conditions of the melanoma patients, 8400 kJ/day (2000 kcal/day) in the form of Nutrison® (Nutricia Holland) were given for 25 days, starting 10 days before the cytostatic treatment.

Samples

Twenty four-hour specimens of urine were collected from melanoma patients and from healthy volunteers. Ten ml aliquots were then stored without any preservation at −20 °C until use.

Hydrolysis, extraction and derivatization

To 2 ml of urine, 500 µl of 2.5 mol/l sodium acetate buffer pH 6.2 and 100 µl of *Helix pomatia* juice were added. The solution was bubbled with nitrogen, the tubes tightly closed and placed in a shaking water bath at 37 °C for 90 min. To each hydrolysed urine sample, a mixture of home-made deuterium labelled indolic substances (300 µl) was added, containing approximately 160 pmol 5,6-dihydroxyindole, 890 pmol 5-hydroxy-6-methoxyindole, 400 pmol 6-hydroxy-5-methoxyindole, 560 pmol 5-hydroxy-6-methoxyindolyl-2-carboxylic acid, 190 pmol 6-hydroxy-5-methoxyindolyl-2-carboxylic acid and 60 pmol 5,6-dimethoxyindolyl-2-carboxylic acid. The samples were saturated with NaCl and extracted with 2 × 4 ml of diethyl ether. Pooled extracts were dried over anhydrous sodium sulphate and evaporated to dryness at 40 °C under a stream of nitrogen.

Derivatization was carried out by the addition of 50 µl of hexafluoroisopropanol followed by 100 µl of pentafluoropropionic anhydride. The tubes were then heated in a heating block at 60 °C for 10 min. After evaporation under a stream of nitrogen, the residue was dissolved in 20 µl of ethyl acetate containing 5% of pentafluoropropionic anhydride.

Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry was performed on a Varian 3700 gas chromatograph interfaced to a Varian MAT 44 S mass spectrometer with an open-split coupling. Data were recorded on a Varian MAT SS 200 data system. The gas chromatograph contained a 25 m × 0.25 mm (i. d.) glass capillary column coated with SE-54 (Franzen Analysen-Technik, Bremen, West Germany). Injector temperature was set at 240 °C, interface temperature at 250 °C and source temperature

at 200 °C. Helium flow rate was 1.8 ml/min and ionization energy 180 eV. The oven temperature was programmed from 150 to 230 °C at 10 °C/min. The reagent gas (methane) pressure was 600 µbar.

Quantification

The concentration of the indolic compounds was obtained by calculating the peak-area ratios of the respective "pseudomolecular" $[M + 1]^+$ ions of labelled and non-labelled compounds and comparing them to those obtained from known ratios plotted in calibration curves using linear regression analysis (PFP = pentafluoropropionyl, HFIP = hexafluoroisopropyl derivative, $-d_0$, $-d_2$, $-d_4$, $-d_5$ = number of deuterium atoms in the molecule).

The following ions were monitored:

m/z 588 corresponding to the $[M + 1]^+$ of 5,6-dihydroxyindole(PFP)$_3$-d_0
m/z 590 corresponding to the $[M + 1]^+$ of 5,6-dihydroxyindole(PFP)$_3$-d_2
m/z 456 corresponding to the $[M + 1]^+$ of 5-hydroxy-6-methoxyindole(PFP)$_2$-d_0 and 6-hydroxy-5-methoxyindole(PFP)$_2$-d_0
m/z 460 corresponding to the $[M + 1]^+$ of 5-hydroxy-6-methoxyindole(PFP)$_2$-d_4 and 6-hydroxy-5-methoxyindole(PFP)$_2$-d_4
m/z 504 corresponding to the $[M + 1]^+$ of 5-hydroxy-6-methoxyindolyl-2-carboxylic acid(HFIP-PFP)-d_0 and 6-hydroxy-5-methoxyindolyl-2-carboxylic acid(HFIP-PFP)-d_0
m/z 509 corresponding to the $[M + 1]^+$ of 5-hydroxy-6-methoxyindolyl-2-carboxylic acid(HFIP-PFP)-d_5 and 6-hydroxy-5-methoxyindolyl-2-carboxylic acid(HFIP-PFP)-d_5
m/z 372 corresponding to the $[M + 1]^+$ of 5,6-dimethoxyindolyl-2-carboxylic acid(HFIP)-d_0
m/z 380 corresponding to the $[M + 1]^+$ of 5,6-dimethoxyindolyl-2-carboxylic acid(HFIP)-d_8

The position of deuterium atoms in the indole molecules was described earlier (9).

Results

As shown in figure 1, the method used gave a good separation of all eumelanin-related indolic compounds, which could therefore be measured in a single chromatographic run. The concentration of each substance was calculated by using its own internal standard and calibration curve. In the concentration range of 0.25—25 nmol/l, the coefficient of variation of the method was satisfactory (2.5—6.0%). However, when normal urine samples and those containing extreme concentrations of indoles were analysed, the coefficient of variation was found to be higher than 10%. In the latter case, quality control could be improved by prior dilution of urine. The recoveries of individual indolic eumelanin-related compounds added to urine samples before their processing were in the range of 80—120%.

The excretion values of indolic eumelanin-related compounds in four melanoma patients before, during and after the therapy are shown in figures 2—5.

Discussion

Although the group of eumelanin-related indolic substances consists of seven indolic compounds, it appears that not all of them are suitable for routine laboratory determination. In general, the main eumelanin precursors, namely 5,6-dihydroxyindole and 5,6-dihydroxyindolyl-2-carboxylic acid, are known to

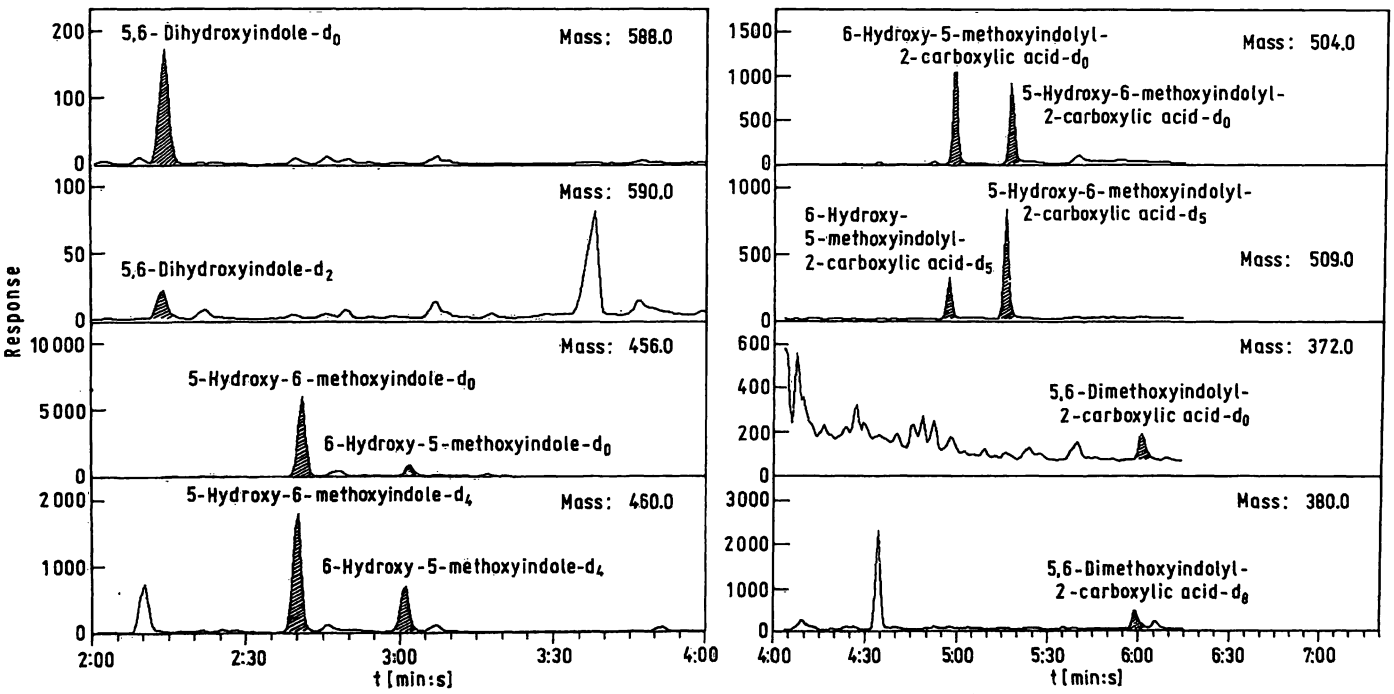

Fig. 1. Mass fragmentograms obtained from a derivatized extract of melanotic urine to which deuterated internal standards were added.

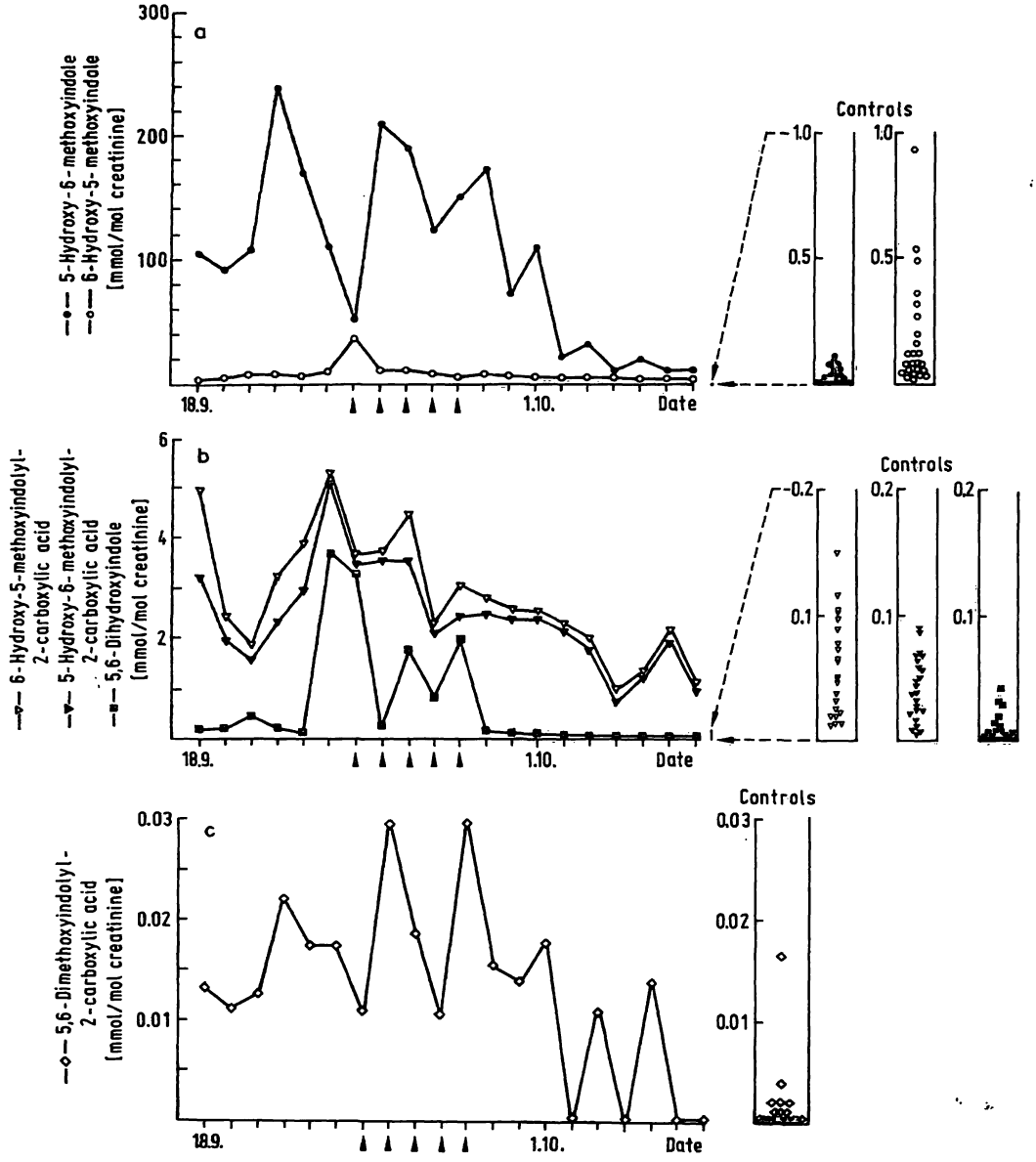

Fig. 2a–c. The urinary excretion of six eumelanin-related compounds by patient K., a woman aged 59, admitted with diagnosis subungual melanoma. Treatment: surgical. In August 1982, generalization of the process with high tumour burden (pulmonary metastases). The arrows indicate the days of the treatment.

readily undergo oxidation and polymerization. Their O-methylation and/or conjugation with sulphate or glucuronate at least partly protect them against the mentioned processes. However, our experience has shown that 5,6-dihydroxyindolyl-2-carboxylic acid is excreted in an unconjugated form and may therefore initiate secondary formation of melanin in melanotic urine during collection and storage. That is why the measured concentration of this substance in urine may not be related solely to its production rate, but may also be dependent on other conditions, such as pH of the urine or the presence of oxidative or reductive substances. In other words, the instability of this eumelanin precursor would make the interpretation of its concentration values unreliable. That was the reason why 5,6-dihydroxyindolyl-2-carboxylic acid was not included in our measurements.

Although 5,6-dihydroxyindole is reported to be excreted as a sulphate conjugate (10), and thus partially protected against polymerization and oxidation, its determination is accompanied by several structurally related difficulties:

(a) the compound is partially oxidized and polymerized during the hydrolysis,

(b) a small difference in the masses of the deuterated (d_2) and natural (d_0) analog causes partial quantitative overlap of measured ions and results in a non-linear calibration curve, and

(c) the instability of deuterated and non-deuterated 5,6-dihydroxyindole during storage of the standard solutions used for calibration and standard addition.

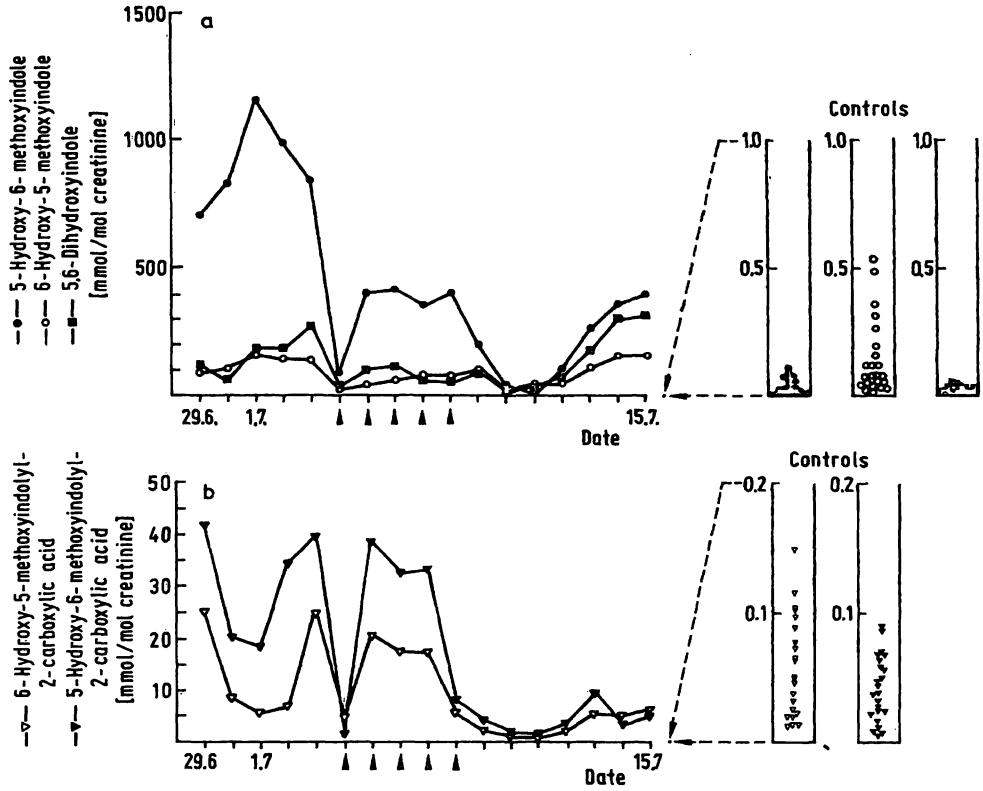

Fig. 3 a—b. The urinary excretion of four eumelanin-related compounds by patient St., a woman aged 24, admitted with diagnosis skin melanoma (shoulder + lymph. nodes). Treatment: surgical and immunotherapy. In April 1981, generalization of the tumour with high tumour burden (pulmonary metastases). The arrows indicate the days of the treatment.

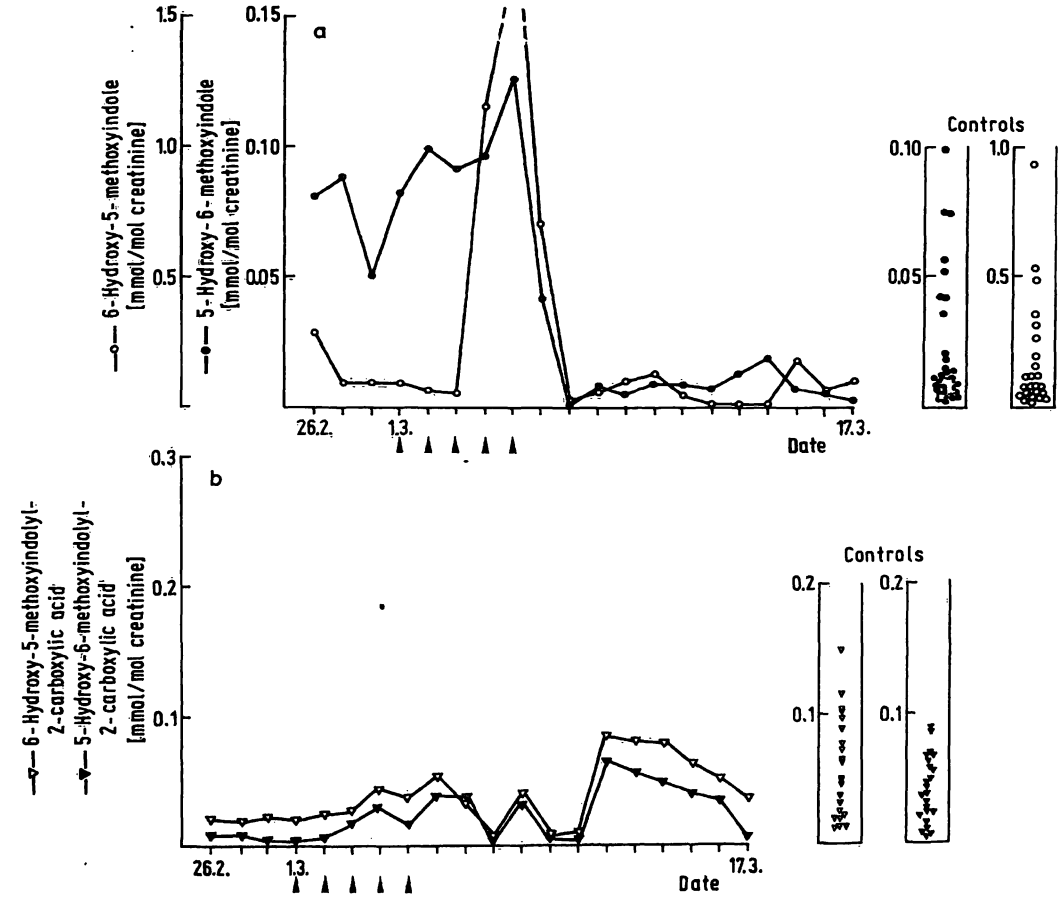

Fig. 4 a—b. The urinary excretion of four eumelanin-related compounds by patient Si., a man aged 38, admitted with diagnosis skin melanoma (the back side of the trunk). Treatment: surgical. In February 1982, tumour progression with low tumour burden (pulmonary metastases). The arrows indicate the days of the treatment.

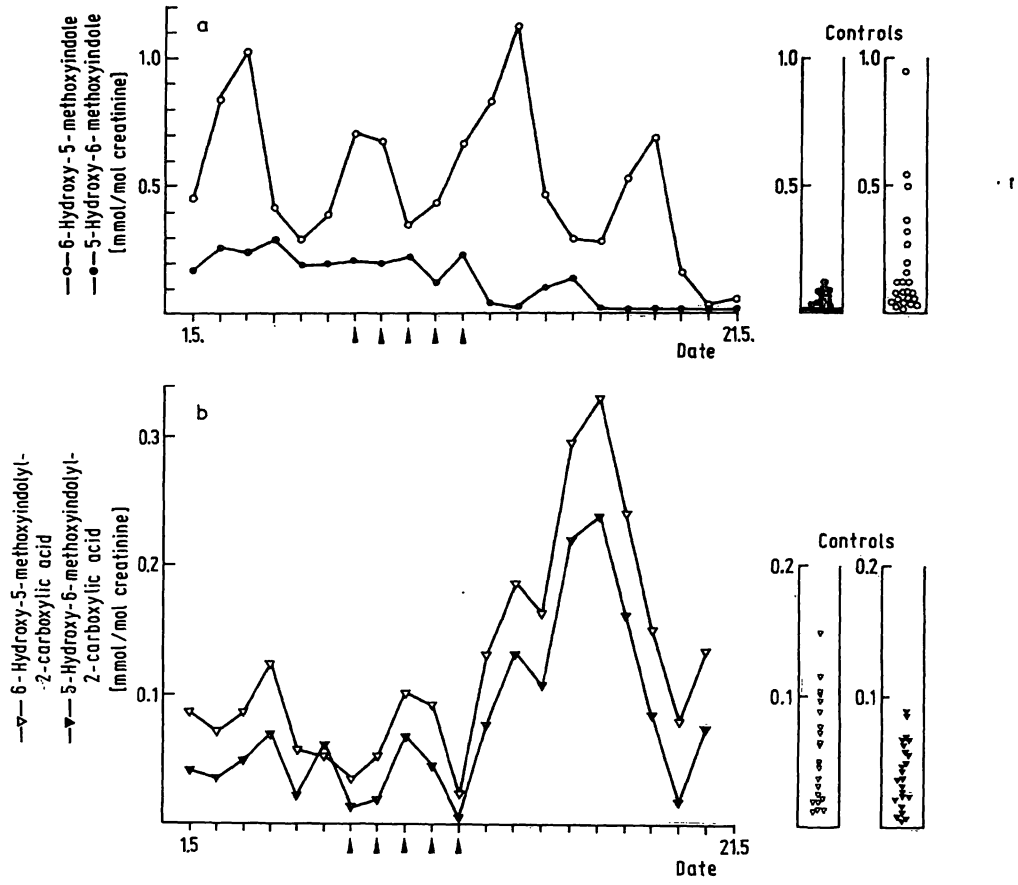

Fig. 5a–b. The urinary excretion of four eumelanin-related compounds by patient S. H., a woman aged 42, admitted with diagnosis skin melanoma (leg). Treatment: surgical. In April 1982, generalization of the disease with high tumour burden (pulmonary metastases). The arrows indicate the days of the treatment.

In view of these problems, the determination of 5,6-dihydroxyindole also does not seem to be suitable for routine purposes.

The recently discovered 5,6-dimethoxyindolyl-2-carboxylic acid (7) was present in normal urine in very low, sometimes undetectable amounts. Although this substance is interesting from the biochemical point of view, the small differences in its excretions in normal and melanotic urine (see fig. 2) make its determination less useful.

From the foregoing it can be seen that O-methylated indoles are apparently more suitable metabolites for routine measurements because of their relative stability. As shown in the follow-up curves of the melanoma patients, 5-hydroxy-6-methoxyindole consistently exhibited the highest differences between pre- and post-therapeutic stadia. It appears that this substance is the most sensitive indicator of increased eumelanin production for pigmented malignant melanoma.

5-Hydroxy-6-methoxyindolyl-2-carboxylic acid and 6-hydroxy-5-methoxyindolyl-2-carboxylic acid were found to be excreted in a pathological range, only if the excretion of the 5-hydroxy-6-methoxyindole was extremely elevated. In other cases, their excretion values were in the normal physiological range, so that they have limited value for the detection of the increased eumelanin production. Our measurements also showed a similar excretion of both isomeric acids, so it appears to be sufficient to measure only one of them.

Some patients exhibited a decrease of 5-hydroxy-6-methoxyindole with a temporal overshoot of isomeric 5-hydroxy-6-methoxy- and 6-hydroxy-5-methoxyindolyl-2-carboxylic acid excretion after the treatment (see fig. 5). This fact raises the question of whether carboxylic and non-carboxylic eumelanin-related compounds share the same mode of generation, cell compartmentalization, and excretion from the organism. Further investigations aimed at interpreting the behaviour of each eumelanin-related metabolite will be necessary.

Since the eumelanin-related substances are specific metabolites of melanocytes, it is obvious that their excretion might be dependent on the amount of these melanin-forming cells as well as on their metabolic, namely melanogenic activity. That is why the decrease of the excretion of the eumelanin-related indoles after the treatment does not necessarily mean the reduction of the tumour mass, but may only indicate metabolic changes in the cellular metabolism of the melanocytes. The long-term follow-up may show whether these changes are transient or permanent, in other words, whether the treatment was effective. Correlation with clinical picture and repetitive measurements of the eumelanin-related compounds are therefore advisable.

In some cases, the high tumour load is not accompanied by an increased excretion of the eumelanin-related metabolites (see fig. 5). This dissapointing fact can be explained by

(a) a low melanogenic activity of the tumour cells and/or by

(b) the existence of an alternative mode of excretion of the indolic metabolites (via bile).

A further investigation must be carried out to clarify the real cause of this discrepancy and to shed more light on the statement, made by *Eppinger* as early as 1910 (11), that so-called melanogenuria appears only after the development of liver metastases.

References

1. Agrup, G., Agrup, P., Andersson, T., Hafström, L., Hansson, C., Jacobsson, S., Jönsson, P.-E., Rorsman, H., Rosengren, A.-M. & Rosengren, E. (1979) Acta Dermatovenerol. (Stockh.) *59*, 381–388.
2. Graef, V. & Paul, E. (1982) Br. J. Dermatol. *106*, 53–57.
3. Jönsson, P.-E., Agrup, G., Arnbjörnsson, E., Hafström, L. O. & Rorsman, H. (1980) Cancer *45*, 245–248.
4. Duchon, J. & Matous, B. (1967) Clin. Chim. Acta *16*, 397–402.
5. Pavel, S., Muskiet, F. A. J., Budesinska, A. & Duchon, J. (1981) Tumori *67*, 325–332.
6. Pavel, S., Muskiet, F. A. J., Nagel, G. T. & Duchon, J. (1981) Sborn. Lek. *83*, 121–127.
7. Pavel, S., Elzinga, H., Muskiet, F. A. J. & Wolthers, B. G. (1983) Acta Dermatovenerol. (Stockh.) *63*, 340–343.
8. Pavel, S. & Muskiet, F. A. J. (1983) In: Human Tumour Markers; Biological Basis and Clinical Relevance (Nierburgs, H. E., Birkmayer, G. D. & Klavins, J. V., eds.) Alan R. Liss Inc. (New York) pp. 311–316.
9. Pavel, S. & Muskiet, F. A. J. (1983) J. Lab. Comp. Radiopharm. *20*, 101–110.
10. Pavel, S., Boverhof, R. & Wolthers, B. G. (1984) J. Invest. Dermatol. *82*, 577–579.
11. Eppinger, H. (1910) Biochem. Z. *28*, 181.

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